Murine Keratinocyte Cultures Grown at the Air/Medium Interface Synthesize Stratum Corneum Lipids and "Recycle" Linoleate During Differentiation

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In a recent investigation we showed that murine keratinocyte cultures grown at the air/medium interface in the presence of dermis exhibit morphologic differentiation comparable to that seen in vivo, including the formation of lamellar granules and stratum corneum intercellular lipid lamellae. In the present study, lifted cultures were found to more closely reproduce the lipid composition of the parent epidermal tissue than submerged cultures grown on plastic. In addition, the specific fatty acid profile of individual lipid classes in lifted cultures was, in general, remarkably well maintained in vitro. Acylceramides, which are highly enriched in linoleic acid in vivo, remained enriched in vitro; however, the linoleic acid content of the cultures was substantially lower than that in vivo, confirming previous reports of the relative essential fatty acid deficiency of standard culture media.

As the lifted cultures differentiated over time, the lipid composition changed to reflect the formation of a stratum corneum with its different complement of lipids. Label from [U-^{14}C]linoleic acid was specifically incorporated into linoleate-containing lipids during short pulses in both submerged and lifted cultures. Changes in label distribution over a long chase period in lifted cultures indicated that linoleate was transferred from phospholipids to ceramides, providing evidence for the "recycling" of essential fatty acids in epidermis.
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A large body of evidence supports the concept that stratum corneum intercellular lipids, arranged in multiple sheets of bilayer membranes, are the structural basis for the epidermal permeability barrier [1–8]. These bilayer sheets arise from the fusion of stacks of membranous disks that are extruded from lamellar granules (LG) into the intercellular spaces at the junction of the stratum granulosum and stratum corneum [4–8]. LG disks are thought to be formed from flattened lipid vesicles [8], thus consisting of two lipid bilayers in close apposition. The origin of these vesicles remains unknown, but it has been suggested that they are synthesized in the Golgi apparatus of the upper spinous and granular cell layers [9]. While the bounding membrane of LG appears to be a typical trilaminar membrane composed primarily of phospholipids, the stacked membrane lamellae within the granule are enriched in ceramides [10], especially the linoleic acid (LA)-rich acylglucosylceramides (AGC) and acylceramides (AC) which are unique to epidermis [11].

The origin of stratum corneum lipids from LG results in a lipid composition that is radically different from that of the living cell layers of the epidermis [12–14]. The composition is quite unusual, consisting of 40% ceramides in addition to cholesterol, free fatty acids (FFA), and cholesteryl sulfate [12–16]. Phospholipids and glucosylceramides (GC), among the most abundant lipids of the viable epidermis, are present in extremely small quantities in the stratum corneum [12,14,15]. Clearly, the transition from a living epidermal cell to a nonviable corneocyte involves complex changes in both the composition and structure of lipid membranes.

AC and AGC are believed to have a specific function in the stacking and stabilization of lipid bilayers in LG, and AC may perform a similar function in the stratum corneum [17,18]. In systemic LA deficiency, the characteristic increase in cutaneous water loss correlates with a decrease in the LA content of AGC and AC [19–21], supporting a direct role for the LA-rich sphingolipids in the maintenance of stratum corneum barrier function [19–22]. Although the epidermis actively engages in de novo synthesis of fatty acyl groups from acetate [23], the LA used in 0-acylsphingolipid synthesis must be obtained directly from the circulation or from other epidermal lipids.

While various keratinocyte culture techniques have been used extensively in the study of protein modifications during differentia-

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Abbreviations:
AC: acylceramide
AGC: acylglucosylceramide
EFA: essential fatty acids
FAME: fatty acid methyl esters
FBS: fetal bovine serum
FFA: free fatty acids
GC: glucosylceramides
GLC: gas-liquid chromatography
LA: linoleic acid
LG: lamellar granule
PBS: phosphate-buffered saline
TLC: thin layer chromatography

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tion [24], the study of lipid metabolism in such cultures is relatively recent. We previously reported on the lipid composition of murine keratinocyte cultures grown on plastic submerged in culture medium [25]. While a full complement of epidermal lipids was present in the cultures, the composition indicated partial differentiation, and, as is true with most culture systems of this type, morphologic differentiation was minimal. Specifically, LG and a stratum corneum were not formed. We recently reported on a lifted murine keratinocyte culture system which closely reproduces in vivo differentiation, including the formation of LG, the extrusion of their contents, and the formation of stratum corneum intercellular lamellae [26]. In this study we report on the lipid and fatty acid composition of these cultures. Since LA is crucial to epidermal barrier function, is most likely of structural importance in the assembly of epidermal membrane structures, and is normally obtained from exogenous sources, we have examined the incorporation of [U-^{14}C]LA into culture lipids, and have followed its redistribution during differentiation of lifted cultures.

MATERIALS AND METHODS

Keratinocyte Cultures Neonatal BALB/c mice were obtained from a breeding colony housed in the University of Iowa Animal Care Facility and fed Purina 5008 rodent chow. Primary neonatal mouse keratinocyte cultures were established as previously described [26]. A basal-cell-enriched fraction was isolated as described by Marcelo et al [27]. The basal-cell-enriched fraction was resuspended in growth medium (M-199, modified, Earle's salts, with glutamine, Flow Laboratories, McLean, VA) + 10% fetal bovine serum (FBS, Hyclone, Sterile Systems, Logan, UT) + 10 μg/ml hydrocortisone + 10 μg/ml insulin + 50 IU/ml penicillin + 50 mg/ml streptomycin. The albumin and LA concentrations in this medium are calculated (based on the analyses supplied with the FBS) to be approximately 35 μM and 1.1 μM, respectively, and the total FFA concentration is 58 μM. Thirty millimeter Millicell wells (Millipore, Bedford, MA) placed in 6-well tissue culture clusters (Costar, Cambridge, MA) were coated with 0.25 ml of 2.0 mg/ml Vitrogen (Collagen Corp., Palo Alto, CA) and gelled at 37°C for 30 min. Basal cells were plated on the gels at a density of 5×10^5 cells/cm^2 and kept at 32°C in a 5% CO_2/95% air atmosphere in a humidified incubator. On the third day after plating, the medium was removed from the top of the cultures, and each culture was placed on mouse dermis spread on a stainless steel mesh elevated slightly from the bottom of a Petri dish. The cultures were fed daily with the medium adjusted to reach the level of the dermis.

In our previous report of lipid composition in submerged cultures grown on plastic, the cultures were plated at a lower density, and a different feeding medium was used. In order to provide a more valid comparison with the lifted cultures, cells were also plated in 100-mm plastic Petri dishes as previously described [25], but at the same density and using the same medium as described above for the lifted cultures.

Cultures were harvested after varying periods of time. The Millicell wells were removed from the dermis, rinsed with phosphate-buffered saline (PBS), and the cultures were peeled off the Millicell membranes using a small glass rod. Generally, 8–10 Millicell wells were pooled for each analysis. Submerged cultures were scraped from the Petri dishes using a teflon policeman. Culture material was placed in glass scintillation vials and frozen at −20°C.

Labeling of Keratinocyte Cultures Lifted cultures were removed from their underlying dermis and placed in the wells of a cluster dish. One μCi of [U-^{14}C]LA (900 mCi/mmol) in 1 ml of growth medium was placed in the well surrounding each Millicell for varying time periods. Cultures were removed from the labeling medium, rinsed several times with PBS, and harvested as described above. A long chase study was conducted in which cultures were labeled for 6 h, rinsed, placed back on the dermis, and grown for varying periods of time before harvesting as described above. Submerged cultures were labeled as described previously [25] with 0.6 μCi [U-^{14}C]LA in 3 ml of medium.

Isolation of Neonatal Mouse Epidermis Neonatal mice were killed under ether anesthesia and immersed in a 60°C water bath for 45 sec. The epidermis was peeled off, placed in a scintillation vial, and frozen at −20°C.

Lipid Extraction Frozen culture material and mouse epidermis were lyophilized and extracted successively with 2:1, 1:1, and 1:2 chloroform:methanol. The extracts were combined and evaporated to dryness under a stream of nitrogen. The lipid residues were redissolved in chloroform:methanol (2:1) for analysis.

Analytical Thin-Layer Chromatography (TLC) Samples were applied in 6-mm-wide lanes scored into 0.25-mm-thick silica gel G-coated 20 × 20 cm glass plates (Alltech, Deerfield, IL). For the nonpolar lipids, the developing solvent was hexane:toluene (1:1, to 20 cm) followed by hexane:ether:acetic acid (70:30:1, twice to 10 cm). Polar lipids, including ceramides, were analyzed using the solvent system chloroform:methanol:water (40:10:1 to 10 cm) followed by chloroform:methanol:acetic acid (190:7:1, twice to 20 cm). Polar lipids were also examined using chloroform:methanol:water (40:10:1, to 15 cm) followed by hexane:ether:acetic acid (70:30:1, to 20 cm). Ceramides were also analyzed using chloroform:methanol:acetic acid (190:9:1, twice to 20 cm). Phospholipid distribution was determined using chloroform:methanol:2-propanol:0.25% KCl:triethylamine (30:9:25:6:18, to 20 cm) [28].

After development, the chromatograms were sprayed with 50% H_2SO_4 and then heated slowly to 220°C on a hotplate to char the lipids. The high sulfuric acid concentration, high temperature, and prolonged period of heating are necessary to produce complete charring of saturated lipids. The chromatograms were cooled and then scanned on a recording photodensitometer (Shimadzu CS-930) that provided automatic peak integration and calculation of percentage composition. Polar lipids and ceramides were identified by comparison on TLC with previously characterized pig epidermal lipids [18,29,30] or with commercial standards. Nonpolar lipids were identified by comparison on TLC with standards obtained from NuChek Prep (Elysian, NY). Phospholipids were identified by comparison on TLC with standards obtained from Sigma (St. Louis, MO). Serial dilutions of appropriate standard lipids were used for quantitation.

Distribution of Label in Culture Lipids The distribution of radioactivity on the chromatograms was determined by use of an automatic TLC-Linear Analyzer equipped with Chroma software (Berthold, Nashua, NH). The total number of cpm in lipid per culture was determined by counting aliquots of the total lipid extract. The number of cpm in individual lipid fractions was then calculated using the percent distribution of label determined by the linear analyzer.

Preparative TLC Preparative TLC was carried out on 0.5-mm-thick silica gel 60 H (E.M. Reagents, Darmstadt, West Germany) coated plates. The chromatograms were visualized by spraying with 2′,7′-dichlorofluorescein (1 mg/ml in 95% ethanol) and then viewing under UV light. Fluorescent bands containing the lipids of interest were scraped from the plate and eluted from the silica with chloroform:methanol:water (50:50:1). Total polar lipids (including ceramides), FFA, and triglycerides were isolated using the nonpolar solvent system detailed above. AC was isolated from polar lipids using chloroform:methanol:acetic acid (190:9:1, twice to 20 cm). The origin fraction from the AC separation was collected, and a total phospholipid fraction was isolated using chloroform:methanol:water (40:10:1, to 20 cm).

Preparation of Fatty Acid Methyl Esters (FAME) Triglycerides were hydrolyzed using 1M NaOH in 90% methanol at 60°C for 1 h. Liberated fatty acids were methylated with 10% BCl_3/methanol at 60°C for 1 h. FAME were extracted into hexane, isolated by preparative TLC using toluene (to 20 cm), and dissolved in hexane. FFA from epidermis and cultures were methylated, and the FAME isolated as above. Phospholipids and AC were hydrolyzed

using methanol:chloroform:10M NaOH (7:2:1) at 40°C; the phospholipids for 1 h, the AC for 0.5 h. The solution was acidified and the liberated fatty acids were extracted into chloroform. The fatty acids were isolated by preparative TLC and converted to methyl esters as described for triglycerides.

Gas Liquid Chromatography (GLC) of FAME GLC was carried out on a 50-m vitreous quartz capillary column with CP SIL 88 stationary phase (Chrompack Inc., Bridgewater, New Jersey) using a Varian 3760 gas chromatograph. An initial temperature of 160°C was maintained for 5 min followed by a 5°/min increase to 220°C which was maintained until all peaks had eluted. Chromatographic peaks were identified by comparison with FAME standards (Nu-Chek Prep). Peak integration and calculation of percentage composition were performed by a Varian CDS 111 electronic integrator.

RESULTS

Lipid Composition In Table I, the lipid composition of lifted keratinocyte cultures at various time points after plating is compared with the lipid compositions of freshly isolated neonatal mouse epidermis and submerged keratinocyte cultures. Figures 1*a* and *b* show thin-layer chromatograms of polar and nonpolar lipids, respectively, in mouse epidermis, 14-d lifted cultures, and 7-d submerged cultures. Day 7 submerged cultures were selected for comparison because they appeared to be maximally differentiated; when culture time was extended to 14 d, increases in the proportions of phospholipids and triglycerides, and decreases in all of the ceramides were seen (data not shown). All of the lipid classes were present in submerged plastic-grown cultures, but the lipids most indicative of differentiation (AC, other ceramides, and FFA) were present in small quantities. GC and phospholipids, lipids associated with a relatively undifferentiated state, were more abundant. Day 3 lifted cultures have actually not yet been lifted to the air/liquid interface, but they differ from the plastic-grown submerged cultures in that they are plated on collagen and are fed from both the undersurface and top of the culture. Phospholipids and GC were also relatively abundant in these cultures, but their ceramide and FFA contents were higher than those of the plastic-grown cultures. As the lifted cultures differentiated from day 3, when they have only a few cell layers, to day 14, when they are fully cornified [26], the relative proportions of phospholipids and GC decreased, and the relative proportions of ceramides and FFA increased.

The overall lipid composition of the lifted cultures between days 10 and 14 was quite similar to that seen in vivo. Lifted cultures had slightly lower phospholipid and FFA contents, and increased ceramide and triglyceride contents compared to the intact epidermis. However, the lifted cultures much more closely reproduced the lipid composition of the intact epidermis than the submerged, plastic-grown cultures.

The ceramide pattern in the lifted cultures was somewhat different from the pattern in the intact epidermis (Fig 1*a*). The band running immediately behind ceramide 2 in the cultures was not present in the epidermis, and we have elected to call this lipid ceramide 2′ until it is further characterized. The proportion of ceramide 2 in the cultures was greater than that in the epidermis. In intact epidermis, monoglycerides were a prominent lipid component, running behind ceramide 2 in the polar solvent systems. Monoglycerides were identified by comparison of their TLC mobility with standard compounds, and by the production of fatty acids as the single charrable product on mild hydrolysis (data not shown). The monoglyceride fatty acids were highly saturated, containing 7% 22:0, 5% 23:0, 76% 24:0, 6% 25:0, and 4% 26:0. Culture

Table I. Lipid Composition of Mouse Epidermis, Lifted Cultures, and Submerged Cultures

Lipid	Mouse Epidermis[a]	Lifted Cultures				Submerged Cultures Day 7
		Day 3	Day 7	Day 10	Day 14	
Total polar	50.0[b]	60.6	52.2	50.3	40.9	64.4
Origin + PLs	22.3	40.0	32.6	23.5	10.7	49.2 ± 5.1[c]
Origin	0.3 ± 0.1	2.3 ± 1.2	1.0 ± 0.3	0.5 ± 0.1	0.6 ± 0.1	
Sphingomyelin	2.6 ± 0.5	5.5 ± 0.6	4.0 ± 1.0	2.4 ± 0.5	1.3 ± 0.4	
PC	9.7 ± 1.7	18.4 ± 0.2	13.1 ± 1.9	10.1 ± 2.2	5.0 ± 0.9	
PS	2.9 ± 0	2.9 ± 1.5	2.4 ± 0.7	2.1 ± 0.2	0.7 ± 0.2	
PI	1.0 ± 0.1	1.8 ± 0.3	1.6 ± 0.2	0.9 ± 0.3	0.5 ± 0.2	
PE	5.8 ± 0.2	9.1 ± 2.3	10.5 ± 0.7	7.5 ± 0.7	2.6 ± 0.7	
GC + Lipid "Y"[d]	1.1 ± 0.2	4.9 ± 0.5	2.4 ± 0.6	2.9 ± 0.3	1.3 ± 0.5	7.2 ± 1.4
AGC	0.7 ± 0.1	0.3 ± 0.1	0.6 ± 0.3	1.1 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
AC	4.1 ± 0.7	0.6 ± 0.6	1.4 ± 0.6	3.1 ± 0.4	3.8 ± 0.9	0.8 ± 0.2
Other ceramides	11.5	14.8	15.2	19.7	24.8	6.8
Cer 2	8.0 ± 0.9	10.4 ± 0.5	9.2 ± 0.7	11.4 ± 1.3	15.5 ± 0.9	4.1 ± 1.2
Cer2′	—	2.4 ± 0.3	3.5 ± 0.5	5.0 ± 1.4	4.8 ± 0.9	1.6 ± 0.7
Cer 3	2.1 ± 0.2	1.6 ± 0.1	2.1 ± 0.4	2.9 ± 0.4	3.3 ± 0.7	0.8 ± 0.1
Cer 4	0.9 ± 0.1	—	trace	0.1 ± 0.1	0.6 ± 0.5	—
Cer 5	0.5 ± 0.2	0.4 ± 0.3	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.5	0.3 ± 0.1
Monoglycerides + 7-OH cholesterol[e]	8.9 ± 1.4					
Total Nonpolar	50.1	39.0	47.1	49.0	59.4	35.5
Cer esters + ?[f]	2.3 ± 0.3	2.7 ± 1.1	2.4 ± 0.3	4.2 ± 0.2	6.4 ± 1.0	1.2 ± 0.6
Sterols	23.5 ± 0.8	28.8 ± 0.9	32.5 ± 2.9	29.4 ± 1.2	30.8 ± 5.3	23.3 ± 0.1
Fatty Acids	11.5 ± 0.4	5.1 ± 1.0	4.9 ± 1.2	6.1 ± 0.8	7.7 ± 0.7	1.9 ± 0.2
Triglycerides	6.5 ± 0.1	0.7 ± 0.6	5.2 ± 3.6	6.0 ± 2.0	10.4 ± 2.6	7.9 ± 0.9
Sterol monoesters	6.3 ± 0.3	1.7 ± 0.2	2.1 ± 0.3	3.3 ± 0.3	4.1 ± 0.1	1.2 ± 0.6
Unidentified	1.3 ± 1.3	—	—	—	—	—
μg lipid per culture	—	103	210	295	332	—
	N = 2	N = 2	N = 3	N = 2	N = 3	N = 2

[a] Excluding the largely sebaceous wax diesters.

[b] Percent of total lipid weight, mean ± range for N = 2, mean ± SD for N = 3.

[c] The PL class composition was not determined in these two experiments; however, our previous study of submerged cultures [25] showed a PL class composition similar to that of epidermis except for a slight increase in the proportion of sphingomyelin.

[d] Unidentified lipid with mobility between PE and the more polar GC in the polar solvent systems (present in small quantities).

[e] 7-hydroxycholesterol is identified by its mobility in comparison to a commercial standard and by the blue color produced on spraying with 50% H_2SO_4.

[f] A large portion of this fraction consists of ceramide esters [16], identified by the production of FA and ceramides on hydrolysis.

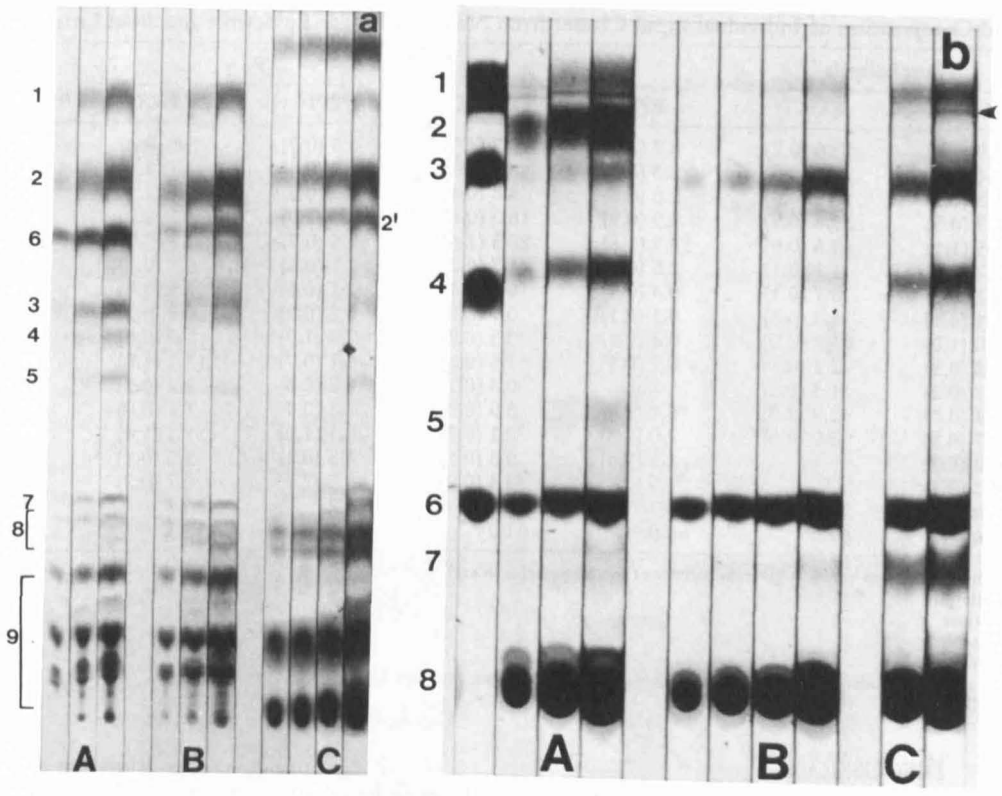

Figure 1. TLC of lipids from mouse epidermis, plastic-grown, submerged keratinocyte cultures, and lifted keratinocyte cultures. *a:* Polar lipids analyzed using the solvent system chloroform : methanol : water (40 : 10 : 1, to 10 cm) followed by chloroform: methanol : acetic acid (190 : 7 : 1, twice to 20 cm). *Lane A:* mouse epidermis; *lane B:* lifted cultures; *lane C:* plastic-grown, submerged cultures. *Lanes A,B,* and *C* contain roughly equal amounts of polar lipid. *Lane C* is from a separate chromatogram: the phospholipid pattern is somewhat different from that in the other two lanes, and some nonpolar lipids are visible at the top of the chromatogram. *1,2,2',3,4,* and *5* are the ceramides (ceramide 1 = AC); *6:* monoacylglycerols + 7-hydroxycholesterol; *7:* AGC; 8 = other GC + lipid "Y"; *9:* phospholipids. *b:* Nonpolar lipids analyzed using the nonpolar solvent system (see text). *Lane A:* mouse epidermis; *lane B:* plastic-grown, submerged cultures; *lane C:* lifted cultures. *Lanes A,B,* and *C* contain roughly equal amounts of lipid. *1:* sterol monesters; *small arrow:* artifact; *2:* sebaceous wax diesters; *3:* triglycerides; *4:* FFA; *5:* unidentified; *6:* cholesterol; *7:* ceramide esters + ?; *8:* polar lipids.

ceramide 2', which we initially thought might be monoglycerides with a different fatty acid chain length distribution, was not affected by mild hydrolysis. The faint band which ran immediately behind culture ceramide 2' (Fig 1*a*), and did not always separate from it, was eliminated by hydrolysis and most likely represents a small amount of culture monoglycerides. 7-Hydroxycholesterol comigrated with monoglycerides in the polar solvent systems, but is present in relatively small quantities in epidermis. Ceramide 3 in the lifted cultures formed a broader band than ceramide 3 in the epidermis, and ceramide 4 was present in smaller and more variable quantities (ceramide 4 is not visible in Fig 1*a*). Ceramide 5 was present in similar proportions in epidermis and lifted cultures. The pattern of ceramides in the submerged plastic-grown cultures was similar to that of the lifted cultures (Fig 1*a*), although smaller amounts were present (Table I). The chromatogram in Fig 1*b* demonstrates the increased proportions of sterol monoesters, FFA, and ceramide esters in the lifted cultures compared to the submerged cultures.

Fatty Acid Composition of Lipids As shown in Table II, the pattern of fatty acids in individual epidermal lipid classes was remarkably well maintained in 14-d lifted cultures. In particular, the relative proportions of saturated and unsaturated fatty acids were almost identical. Both phospholipids and triglycerides contained predominantly short chain ($\leq$18C) fatty acids. The FFA contained a high proportion of long chain ($\geq$20C) species (63.5% in the epidermis and 59.5% in the cultures). AC was highly enriched in LA (18 : 2). The most striking difference in the fatty acid composition of the cultures compared to that of the epidermis was the lower percentage of LA seen in all lipid classes in the cultures. Culture AC was still highly enriched in LA compared to the other lipids, but it had only 50% of the LA content of the epidermal AC. The phospholipids and FFA of the cultures contained 25% of the LA content of the corresponding epidermal lipids, and culture triglycerides contained 50% of the LA content of epidermal triglycerides. Lignoceric acid (24 : 0) was lower in culture phospholipids and AC than in the corresponding epidermal lipids, but was maintained at the same high level in FFA as in vivo.

Eighteen-Hour Pulse Labeling with [U-^{14}C]LA Table III and Fig 2 show the results of labeling 14-d lifted cultures and 7-d submerged plastic-grown cultures for 18 h with [U-^{14}C]LA. Phospholipids and the unidentified lipid "Y" were heavily labeled in both types of cultures. AGC and AC were also labeled in both types of cultures, but 2 and 3 times more label, respectively, were incorporated in the lifted cultures compared to the submerged (Table III). FA and TG were also significantly labeled in both types of cultures. Labeling of sterols and other ceramides, indicating nonspecific incorporation, was quite low after the 18-h pulse.

Redistribution of LA Label During Culture Differentiation The changes in relative specific activity of polar and nonpolar lipids over time after a 6-h LA pulse on day 8 of culture are shown in Figs 3*a* and *b*, respectively. Immediately after the pulse, phospholipids and triglycerides were the most heavily labeled, followed by FFA and AGC. The specific activity of AC was only slightly higher than that of the other nonspecifically labeled ceramides. After a 1-d chase period, LA labeling of phospholipids and FFA decreased, while labeling of the LA-containing ceramides increased. As phospholipid label continued to decrease, AGC specific activity increased to a peak after a 2-d chase and then declined on day 14 after a 6-d chase. AC specific activity rose steadily during culture differentiation, and by day 14 AC had the highest specific activity of the lipids.

Table II. Fatty Acid Composition of Individual Lipid Classes from Neonatal Mouse Epidermis and 14-d Lifted Keratinocyte Cultures[a]

	Phospholipids		AC		FFA		Triglycerides	
FA	EPI[b]	CULT[c]	EPI	CULT	EPI	CULT	EPI	CULT
16:0	12.9 (0.2)	13.6 (0.2)	6.7 (2.5)	12.7 (1.5)	5.3 (0.7)	5.0 (0.4)	23.8 (1.6)	20.1 (1.0)
16:1	5.1 (0.2)	12.3 (0.5)	2.3 (0.8)	8.8 (1.1)	1.2 (0.4)	2.7 (1.3)	4.0 (0.1)	6.7 (0.2)
18:0	11.3 (0.2)	10.9 (0.6)	2.3 (0.8)	4.8 (0.1)	2.7 (0.2)	5.1 (1.1)	9.5 (0.9)	10.0 (0.2)
18:1	27.9 (0.5)	33.8 (0.9)	2.9 (0.9)	18.3 (0.6)	6.9 (2.0)	8.1 (2.2)	31.7 (1.6)	33.3 (1.8)
18:2	14.5 (1.1)	3.6 (0.0)	59.9 (1.8)	28.5 (1.6)	13.5 (0.7)	3.3 (0.7)	14.3 (0.4)	7.5 (0.4)
18:3 + 20:1	0.7 (0.1)	1.1 (0.1)	2.5 (0.2)	1.7 (0.2)	1.1 (0.1)	0.7 (0.1)	3.3 (1.0)	3.0 (0.4)
20:0	1.1 (0.3)	0.7 (0.1)	0.4 (0.1)	0.9 (0.1)	1.1 (0.1)	2.9 (0.6)	1.8 (0.7)	2.3 (0.2)
20:3	1.1 (0.1)	1.3 (0.3)	0.1 (0.1)	0.9 (0.2)	0.2 (0.2)	0.9 (0.1)	0.3 (0.1)	0.7 (0.0)
20:4	7.0 (1.2)	6.8 (0.0)	0.3 (0.1)	2.5 (0.2)	2.3 (1.9)	1.9 (0.8)	0.9 (0.1)	2.8 (0.0)
22:0	2.3 (0.3)	2.1 (0.3)	0.7 (0.1)	1.5 (0.3)	5.1 (0.7)	13.1 (1.8)	0.7 (0.2)	1.5 (0.3)
22:6	1.7 (0.3)	1.3 (0.2)	—	0.3 (0.1)	0.8 (0.6)	0.7 (0.3)	0.5 (0.1)	0.7 (0.1)
23:0	1.1 (0.3)	2.9 (2.0)	6.1 (0.8)	3.9 (0.9)	2.1 (0.4)	1.1 (0.0)	0.7 (0.2)	0.7 (0.0)
24:0	5.7 (0.8)	3.0 (0.6)	7.9 (5.8)	2.1 (0.2)	29.1 (1.2)	31.1 (1.4)	0.9 (0.3)	1.3 (0.3)
25:0	0.2 (0.2)	—	1.1 (0.6)	0.3 (0.1)	4.5 (0.1)	1.1 (0.1)	—	—
26:0	0.3 (0.1)	—	1.5 (0.8)	0.4 (0.0)	18.3 (2.7)	6.7 (0.2)	—	—
Sat[d]	34.9	33.2	26.7	26.6	68.2	66.1	37.4	35.9
Unsat[e]	58.0	60.2	68.0	61.0	26.0	18.3	55.0	54.7

[a] N = 2, the average ± range of the two values is given as percent of total fatty acids in each lipid class.
[b] EPI: neonatal mouse epidermis.
[c] CULT: keratinocyte cultures.
[d] Sat: total saturated fatty acids.
[e] Unsat: total unsaturated fatty acids.
Some of the minor fatty acids and unidentified fatty acids have been omitted; therefore, the total does not equal 100%.

DISCUSSION

Epidermal differentiation is accompanied by profound changes in lipid composition, and in the morphology of specialized lipid structures. The production of LG, the extrusion of their contents, and the formation of the stratum corneum intercellular lipid lamellae are markers of terminal differentiation that do not occur in typical submerged keratinocyte cultures. Although submerged cultures have been adequate for the study of other differentiation-related events, they are not suitable for studying many of the complex processes involved in epidermal lipid metabolism and morphogenesis.

The morphology of the lifted murine keratinocyte culture system used in this study has previously been shown to closely resemble that of its parent epidermal tissue, by both light and electron microscopy [26]. The present results indicate that the lipid composition of the lifted cultures is much closer to the lipid composition of the parent epidermal tissue than is the composition of submerged, plastic-grown cultures. Although all of the epidermal lipid classes are present in submerged cultures, the lack of morphologic differentiation is reflected in the lipid composition, and makes that type of culture a less desirable model for the study of epidermal lipid metabolism.

The changes in lipid composition that occurred as the lifted cultures differentiated recapitulate the changes in composition reported for different layers of the epidermis [12–14]. The relative amounts of phospholipids and GC (components of the living cell layers) decreased, while ceramides and FFA (major components of stratum corneum) increased, as the cultures became more fully cornified. Sterols, which are a major lipid component throughout the epidermis, remained constant. Triglycerides were somewhat elevated in both submerged and fully differentiated lifted cultures compared to the epidermis, but not dramatically. It should be noted that the triglyceride content of normal epidermis is quite low; this reflects the lack of contamination with subcutaneous tissue when the skin is not excised prior to isolation of the epidermis [23]. Between days 10 and 14, the lipid composition of the lifted cultures closely resembled that of the epidermis. Submerged cultures had substantially less AC, ceramides, and FFA, and more phospholipids and GC compared to lifted cultures, indicating a relatively undifferentiated state.

The lipid composition of whole mouse epidermis was similar to that recently reported for whole pig epidermis [23]. An interesting exception was the presence of monoacylglycerols in mouse epidermis. These have been previously reported in mouse epidermis [31], but have not been noted in either pig or human epidermis.

Table III. ^{14}C-Linoleic Acid Incorporation Into Culture Lipids During an 18 h Pulse[a]

	Lifted Cultures		Submerged Cultures	
Lipid	% Total Lipid	% Total Label	% Total Lipid	% Total Label
PL	20.3	87.1	44.1	86.5
GC + "Y"	3.7	6.2[b]	8.5	4.9[b]
AGC	0.9	1.0	0.6	0.5
AC	4.6	0.9	0.6	0.3
Other cer	19.5	0.9	8.7	<0.1
Cer esters + ?	7.4	0.2	1.7	0.3
Sterols	26.3	0.5	23.3	0.8
FA	5.8	0.9	1.7	1.1
TG	6.7	1.9	8.8	5.4
SME	4.8	0.2	1.8	<0.1

[a] The data are from a single experiment in which both lifted cultures and submerged cultures on plastic were grown in parallel. Lifted cultures were labeled on day 14, submerged cultures on day 7. Several cultures of each type were pooled for the lipid analyses.
[b] All of the label is in lipid "Y".

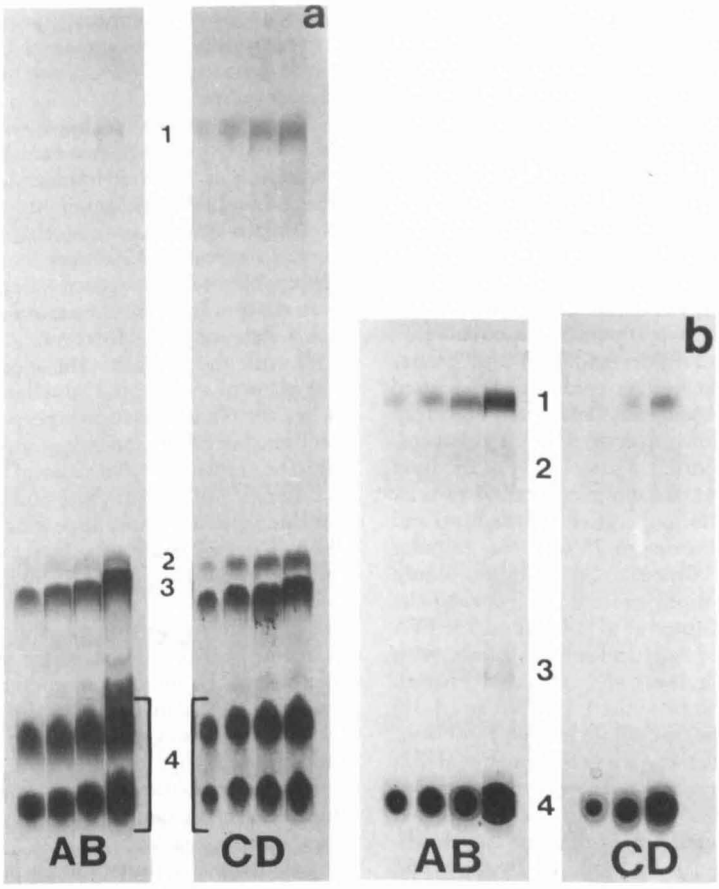

Figure 2. Autoradiograms of chromatographed [U-^{14}C]LA-labeled keratinocyte culture lipids. Cultures were labeled for 18 h prior to harvesting. *a:* Polar lipids analyzed using the solvent system as in Fig 1*a*. *Lanes A* and *B:* plastic-grown, submerged cultures; *lanes C* and *D:* lifted cultures. *Lanes A* and *D* contain roughly equal quantities of lipid; *lanes B* and *C* contain roughly equivalent cpm. 1: AC; *2:* AGC; 3: lipid "Y"; 4: phospholipids. *b:* Nonpolar lipids analyzed using the nonpolar solvent system. *Lanes A* and *B:* plastic-grown, submerged cultures; *Lanes C* and *D:* lifted cultures. *Lanes B* and *D* contain roughly equal quantities of lipid; *Lanes A* and *C* contain roughly equal cpm. *1:* triglycerides; *2:* FFA; *3:* sterols; *4:* polar lipids.

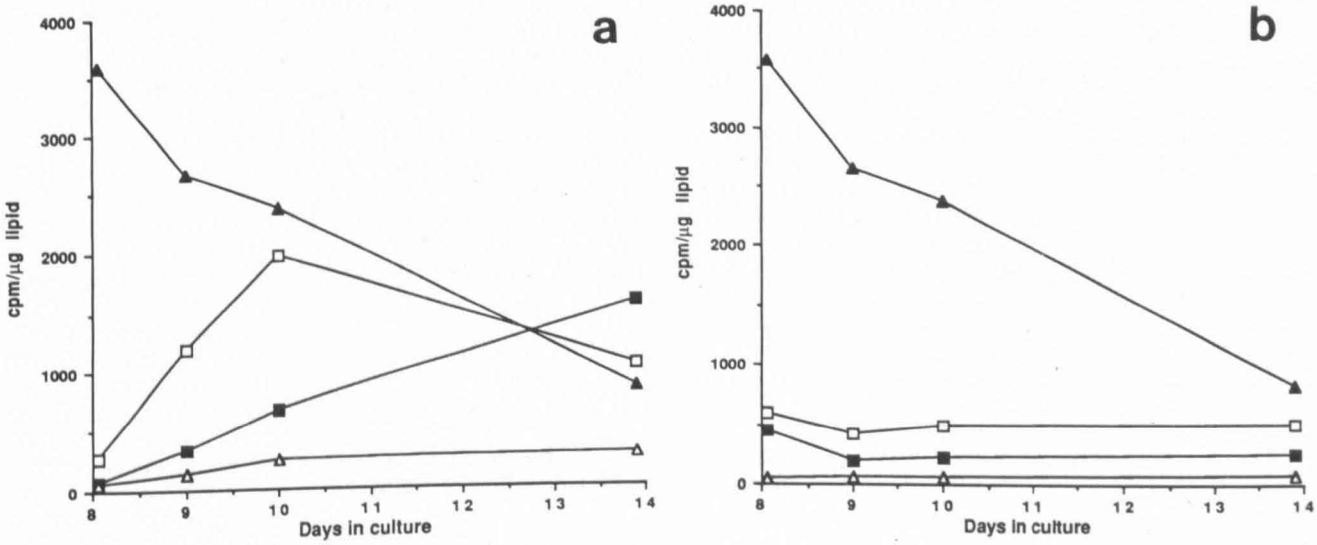

Figure 3. Redistribution of LA label in polar and nonpolar culture lipids during differentiation. Cultures were labeled for 6 h on day 8 of culture (5 d after lifting). Ten cultures were removed immediately after the labeling period for lipid analysis. The rest of the cultures were rinsed, placed back on the dermis, and grown for additional periods of time. On days 9 (24-h chase), 10 (48-h chase), and 14 (6-d chase), ten cultures were removed at each timepoint for lipid analysis. *a:* Polar lipids; *closed triangle:* phospholipids; *open square:* AGC; *closed square:* AC; *open triangle:* other ceramides. *b:* Nonpolar lipids. Phospholipids *(closed triangle)* are shown for comparison. *Open square:* triglycerides; *closed square:* FFA; *open triangle:* sterols.

Their structure and degree of saturation would, however, be compatible with a role in stratum corneum lipid bilayer formation. Monoacylglycerols are not synthesized in significant quantities by the cultured keratinocytes, and it is possible that the increased proportion of ceramides seen in culture represents, in part, an attempt to compensate for the lack of monoacylglycerols.

The only directly comparable published study of lipids in lifted keratinocyte cultures (by Ponec et al, using human cultures [32]) also showed decreases in the proportions of phospholipids and GC and increases in ceramides and FFA with increasing culture time. As in our lifted mouse cultures, the lifted human cultures had less phospholipid and more ceramides and FFA than submerged cultures [32]. Our results, however, differ on several points. Ponec et al [32] found much lower quantities of ceramides and FFA and greater quantities of phospholipids in intact human epidermis (and lifted cultures) than we found in mouse epidermis (and lifted cultures). Although complete lipid compositional data on whole human epidermis have not been previously reported, a prior study of the lipid composition in different populations of human epidermal cells by Lampe et al [14] showed the phospholipid content of the basal and spinous layers to be 45%, with decreases to 25% in the granular layer and about 4% in the stratum corneum. These figures would result in a total epidermal phospholipid content far less than the 66% reported by Ponec et al [32]. Lampe et al [14] found 7% FFA and 3.8% ceramides in the combined basal and spinous layers, with substantial increases in the granular layer and stratum corneum. Again, these figures are quite different from the 1.1% FFA and 4.3% ceramides reported by Ponec et al [32] for whole human epidermis. The reason for the differences between the study by Ponec et al [32] and the one by Lampe et al [14] is unclear. However, the mouse epidermal lipid composition reported here is consistent with that reported by Lampe et al for human epidermal cell populations [14], by Hedberg et al for intact pig epidermis [23], and by Gray and Yardley for pig epidermal cell populations [12].

Of particular interest to us were the LA-rich AGC and AC. Although Ponec et al reported that AGC and AC were not present in submerged human cultures [32], we did detect these lipids in submerged mouse cultures. This difference may be due to the inability of passaged human cells to make AGC and AC. The decreases in these lipids that we found on longer cultivation of the submerged primary mouse cultures is consistent with a loss of synthetic ability. In both the study by Ponec et al [32] and our study, the proportion of AGC increased initially in lifted cultures but declined at 14 d. Relative thinning of the living cell layers on day 14 of culture and beyond was noted by Ponec et al in the lifted human cultures [32] and by us in the lifted mouse cultures (data not shown), and may account for the decline. In both studies, AC increased steadily as the cultures differentiated over time. The pattern of the FFA and the esterified fatty acids of phospholipids, triglycerides, and AC was remarkably well maintained in these cultures. The FFA of the lifted mouse cultures contained the same high proportions of long ($\geq$20C) chain and saturated fatty acids as the FFA of the intact epidermis. This reflects the contribution of the predominantly long chain and saturated FFA of stratum corneum and is another indicator of terminal epidermal differentiation. Epidermal AC was highly enriched in LA, as has now been reported for several species [16,18,20]. The most striking difference in fatty acids between the epidermis and the cultures was the lower LA content of the cultures. This has been reported previously in plastic-grown submerged mouse keratinocyte cultures by us and others [25,33], in submerged and lifted human keratinocyte cultures [32], and in other cell culture types [34,35], and is most likely due to the relatively low LA content of FBS [33–35]. In spite of this deficiency, culture AC remained substantially enriched in LA compared to the other lipid classes; the major replacement fatty acid was oleic acid (18:1), as has been demonstrated in EFA deficient animals [19–21]. The LA deficiency reported by Ponec et al [32] in lifted human cultures was more profound than that seen in these lifted mouse cultures. In their study, total lipid fatty acids, phospholipid fatty acids, and the esterified fatty acids of AGC contained less than 5% of the amount of LA found in the corresponding epidermal lipids. This may be related to the passaging of the human cells prior to the establishment of the lifted cultures, and/or the use of medium containing a lower FBS concentration.

We previously speculated [25] that the LA deficiency seen in submerged cultures grown on plastic might contribute to the lack of formation of lamellar lipid structures. It was previously reported that LG in EFA-deficient-mouse epidermis appeared to be empty or to contain amorphous material rather than the normal stacked lamellar structures [36]. Ponec et al found partially and completely empty LG in lifted human keratinocyte cultures and felt that this was morphologic confirmation of the demonstrated biochemical LA deficiency [32]. However, an early study of EFA deficient mice [37] made the point that the appearance of LG varies depending on the plane of section; the lamellar internal structure is apparent only when the plane of section is perpendicular to the lamellae. Granules sectioned in other planes may appear to contain amorphous material or to be empty. A recent study of EFA deficient pigs [21] did not find a difference in LG structure compared to control pigs; both typical and amorphous/empty appearing LG were present in normal and in EFA-deficient epidermis. Intact stratum corneum intercellular lamellae were also present in deficient animals. This suggests that oleic acid (the usual replacement fatty acid in EFA deficient animals) is capable of supporting membrane bilayer structure in the stratum corneum; however, the water permeability of the membranes is increased [21], and the membranes may be more susceptible to fracture [36]. Williams et al [38] also noted the presence of LG in lifted human keratinocyte cultures but commented that the quantity did not approach that found in vivo. Lamellar lipid structures indistinguishable from those in intact epidermis are present [26] in the moderately LA-deficient lifted murine keratinocyte cultures discussed in this paper.

Labeled acetate incorporation into keratinocyte culture lipids has been used to study rates of lipogenesis and to determine if certain lipids are synthesized in culture [32,38,39]. As an essential fatty acid, LA is not endogenously synthesized and must be obtained from the circulation or from other lipids via a FFA pool or via acyl exchange. Over an 18-h labeling period, both submerged and lifted cultures specifically incorporated label into LA-containing epidermal lipids. AGC and AC in the lifted cultures incorporated a greater proportion of label than in the submerged cultures.

When lifted cultures were pulsed with LA for a short time on day 8 of culture and then chased for 6 d in growth medium, the distribution of lipid label changed dramatically. LA was released from phospholipids and salvaged by incorporation into other LA-containing lipids. LA salvage was demonstrated many years ago by Freinkel and Traczyk [40] using a fetal rat skin organ culture model, but they did not examine incorporation into ceramides. Both AGC and AC acquired large quantities of released label, and the time course of their labeling is of particular interest. It has been proposed that AC is produced as a result of deglycosylation of AGC [41–43]. The low specific activity of AC after the initial labeling period followed by a steady increase during differentiation is consistent with this concept. AC labeling would be expected to increase only as newly labeled AGC was deglycosylated. Although the location of the deglycosylation step is not known, it may occur partly in the LG and partly after extrusion of LG contents into the intercellular space. The higher specific activity of AGC, which is present only in the living cell layers, reflects the rapid turnover of this lipid. AGC is believed to be the precursor, not only of AC, but also of the hydroxyceramide moieties of the corneocyte lipid envelope [15,23]. The decline in AGC specific activity on day 14 of culture may reflect depletion of the specific pool of labeled LA available for synthesis of this molecule. Our time course results are similar to those reported for acylsphingolipid labeling following topical application of labeled LA to rat skin; the results of that study were also interpreted to support a precursor/product relationship between AGC and AC [42].

This culture system is an excellent model for the study of epidermal lipid structures and lipid metabolism, and should be useful for

the study of other aspects of terminal epidermal differentiation which are not adequately reproduced by standard submerged cultures. Further studies are needed to determine if supplementation of culture medium with LA will increase the LA content to the level seen in vivo. Although not required for the assembly of lamellar lipid structures, this may prove important in establishing permeability barrier function equivalent to that seen in vivo. In addition, recent work using a number of cell types indicates that the lipid composition of cell membranes significantly affects a variety of important cell functions including membrane-bound enzyme activity, receptor binding, carrier-mediated transport, and eicosanoid production [44–46], and this will undoubtedly be true for keratinocytes as well.

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